PhD Thesis

The relationship in different habitat types between Hungarian orchid species, their symbiont partners and the abiotic environmental conditions

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Introduction

The Orchidaceae family contains more than 25000 species which are very diverse regarding their colours, morphology and lifestyles. Orchids are vulnerable resulting from antropogen factors and their mutualistic relationship with their pollinators and mycorrhizal fungi. For their effective conservation, it would be necessary to know the essential needs of orchids, their pollinators and their symbiont partners, but only a limited number of studies have addressed the two latter factors.

During germination, the fungal partners provide the essential nutrients for orchid seeds, because the dust-like seeds of orchids contain scant energy reserves. Adult orchids also live in symbiosis with mycorrhizal fungi. The orchid symbiont fungi belong to the form genus *Rhizoctonia (Ceratobasidium*, *Thanatephorus*, *Sebacina*, and *Epulorhiza* genus), but in the last few decades some ecomycorhrizal fungal lineages were also shown to be the mycorrhizal partners of orchids (*Tuber*, *Russula*, *Coprinus*). This strategy occurs mainly in shaded forest habitats, where the orchids can not produce enough nutrients by photosynthesis, and they get organic nutrients from the surrounding trees via mycorrhizal fungi.

The specificity of orchid mycorrhiza is defined with the phylogenetic breadth of the symbiont partners. Generalist orchids are able to form mycorrhiza with several fungal genera or families, which allows them to colonise habitats with various fungal communities. On the contrary, specialist orchids live in symbiosis with only one fungal species or a very narrow clade of fungi, resulting in a very effective nutrient exchange. It is not clear whether the specialist or generalist strategy is better for orchids.

Despite several studies addressing the orchid mycorrhizal fungi of natural habitats, very little is known of the mycorrhizal partners of orchids living in disturbed habitats (e.g. in abandoned mines), especially in Hungary. Evidence is accumulating that environmental factors (e.g. temperature, water supply) affect the composition of fungal community, but no information is available on the effects of these factors on orchid symbiont fungi.

Objectives

The main goals of this study were

1. to identify the fungal partners of orchids living in abandoned mines with molecular methods,
    a. to compare the fungal communities of the investigated abandoned mines,
    b. to compare the fungal communities of abandoned mines and natural habitats,
    c. to investigate the fungal specificity of *Orchis militaris*;
2. to determine the potential symbiont partners of orchids living in forest habitats by means of a hypogeous fungal database,
3. to identify the fungal partners of orchids living in forest habitats with molecular methods;
4. to determine the growing rate of orchid symbiont strains originating from habitats with different water supply, at different temperatures and water activities.

Materials and Methods

Field studies were conducted in three abandoned mines:

- Pusztavám – Cica-homok, abandoned coal mine,
- Székesfehérvár – Sóstó Sand Mine,
- Tokodaltáró - Gete Sand Mine,

and in two forest habitats:

- Algyő – Lúdvári-erdő,
- Szigetcsép oak forest.

Roots of orchid individuals were collected at the investigated habitats as follows:

- abandoned mines (between 2006 and 2009, in the flowering period of orchids)
    - Pusztavám: *Orchis militaris* (7)
    - Székesfehérvár: *Orchis militaris* (1), *Anacamptis coriophora* (2), *Anacamptis palustris* ssp. *palustris* (2) and *Dactylorhiza incarnata* (1)
    - Tokodaltáró: *Orchis militaris* (3), *Dactylorhiza incarnata* (2) and *Epipactis palustris* (1)
- forest habitats (between 2007 and 2008)
    - Algyő: *Epipactis helleborine* agg. (1)
    - Szigetcsép: *Epipactis microphylla* (1) and *Cephalanthera damasonium* (1)

Fungal sequences of the nrITS region were obtained from isolated fungal strains or directly from mycorrhizal root segments. Fungi were identified based on sequences deposited in the NCBI BLAST database. Sequences were aligned and converted with the MAFFT and ALTER programs. Phylogenetic analysis, applying the maximum likelihood criterion, was conducted with RaxML GUI 0.95.

Soil samples were collected and analysed in all of the investigated habitats. A coenological survey was also conducted, and the degradation tolerance and water demand of vascular plants was determined according to the Hungarian Flora Database.

The following data are presented in the hypogeous fungal database: species name, herbarial number, the time of gathering, the gatherer, geographical unit, the settlement, type of the habitat, exposure of the habitat, name of the truffle dog, type of the soil, association and partner plant. A coenological survey was also carried out in the surroundings of all the hypogeous fungi, showing if there was an orchid near the hypogeous fungal fruit bodies. Data were analysed with Microsoft Office Excel 2003.

Members of the *Ceratobasidium* and *Epulorhiza* genera were chosen for the temperature and drought stress experiments. The fungal strains derived from floating mats, from wet habitats and from habitats with variable water supply. The growing rate of the fungal strains was determined on PDA medium, based on the daily measurement of the diameter of the growing colonies. Measurements were executed at seven temperatures (5-35°C) and six water activities (0,988-0,64 a_w). Normality of the obtained data was tested, then the data were compared with oneway-ANOVA or Kruskal-Wallis test at 5% significance.

Thesis

1. 42 sequences were obtained from the roots of orchids living in abandoned mines, including three orchid symbiont genera (*Ceratobasidium*, *Sebacina* and *Epulorhiza*) and four fungal endophyte groups (Coprinaceae, *Fusarium* spp., *Leptodontidium* spp. and Pezizaceae). To our knowledge, this is the first time that members of Coprinaceae are shown out from the roots of photosynthesising orchids.
    a. The most diverse fungal community was found in Székesfehérvár, where three groups of *Rhizoctonia* symbionts and three groups of non-*Rhizoctonia* endophytes were found. In Tokodaltáró the community was slightly less diverse but also diversified (three groups of classical symbionts and one endophyte group), while in Pusztavám it was restricted to only one group of *Rhizoctonia* symbionts and three groups of non-*Rhizoctonia* fungi. It appears that habitats with a more diverse and less disturbed flora have a more diverse symbiont community.
    b. Members of the genus *Epulorhiza* seem to be more frequent in the mines compared to natural habitats, especially for *Epulorhiza* 2 symbionts. This may lend support to the hypothesis that *Epulorhiza* species may be present mostly in disturbed habitats, and tolerate well the unfavourable environmental circumstances.
    c. Our observations indicate that the specificity of *Orchis militaris* is looser than previously assumed. Three *Epulorhiza* 2 clades, *Epulorhiza* 1, *Ceratobasidium* and *Sebacina* symbionts and Coprinaceae, *Fusarium* spp. and *Cadophora* sp, endophytes were obtained from the roots of *Orchis militaris*, confirming that *Orchis militaris* is generalist in terms of specificity.
2. *Tuber excavatum* and *Tuber rapaeodorum* were shown from the roots of forest orchids, the latter for the first time.
3. The data from the hypogeous fungal database support the view that *Tuber* and *Hymenogaster* species could be mycorrhizal partners of orchids. These two fungal genera represent 75% of the hypogeous fungi found in the surroundings of orchids.

4. In the temperature and drought stress experiments, the growing rates of the fungal strains were significantly different at all but some experimental settings, suggesting that the investigated fungal strains adapted to the environmental circumstances of their habitat. However, minor differences from our preliminary expectations suggest that the fungal strains tolerate relatively wide ranges of temperature and water supply.

Conclusions

Orchids live under unfavourable environmental circumstances in the abandoned mines and forests, as there is only limited light in forests and pioneer circumstances in the mines. Non-*Rhizoctonia* fungal partners could be identified in both habitat types from the roots of the investigated orchids, and what is more, only non-*Rhizoctonia* symbionts were obtained in the forest habitats. It has been previously established that *Tuber* species could be real mycorrhizal partners of forest orchids, so the partner switch have passed in this habitat type. It is likely that a previous state of orchid mycorrhizal evolution could be seen in abandoned mines, where orchid roots are colonised by orchid symbionts and endophytes. It is not known whether these endophytes have any positive effect on orchids, but a *Coprinus* species proved to be orchid symbiont, and it has been suggested that some *Fusarium* and *Leptodontidium* species could be in mutualistic relationship with orchids. Taken together it appears that orchids, especially in mines under unfavourable circumstances, associate with several fungal species, as generalists. It is possible that some endophyte fungi could turn into symbiont partners during their evolution.

Orchis militaris seemed to be a specialist according to earlier studies because there were no other symbiont partners available at the investigated habitats. This observation draws attention that environmental circumstances may affect orchids and their symbiont partners. During orchid conservation programs it would be necessary to know not only the symbiont partners of orchids, but also the optimal environmental conditions for them.

Related publications

Ouanphanivanh N, Illyés Z, Merényi Zs, Orczán ÁK, Szigeti Z, Bratek Z (2013) Mycorrhizal fungi and endophytes of orchids living in three Hungarian abandoned mines. Sydowia 65:(2) pp. 245-266.

Illyés Z, **Ouanphanivanh N**, Rudnóy Sz, Orczán ÁK, Bratek Z(2010) The most recent results on orchid mycorrhizal fungi in Hungary. Acta Biologica Hungarica 61:(Supplement 1) pp. 68-76.

Illyés Z, Halász K, Rudnóy Sz, **Ouanphanivanh N**, Garay T, Bratek Z (2009) Changes in the diversity of the mycorrhizal fungi of orchids as a function of the water supply of the habitat. Journal of Applied Botany and Food Quality-Angewandte Botanik 83: pp. 28-36.

Ouanphanivanh N, Zs Merényi, ÁK Orczán, Z Bratek, Z Szigeti, Z Illyés (2008) Could orchids indicate truffle habitats?: Mycorrhizal association between orchids and truffles Acta Biologica Szegediensis 52:(1) pp. 229-232.

Ouanphanivanh N, Illyés Z, Rudnóy Sz, Bratek Z (2007) Hazai *Orchis militaris* élőhelyek orchidea-mikorrhiza gombáinak vizsgálata. Tájökológiai Lapok 5:(2) pp. 325-332. (in Hungarian with English abstract)

Ouanphanivanh N, Illyés Z, Szigeti Z, Bratek Z (2009) A Székesfehérvár-Sóstói felhagyott homokbányában élő orchideák szimbionta gombapartnereinek azonosítása. 8. Magyar ökológus kongresszus, Szeged, 2009. augusztus 26-28. In: Körmöczi L (szerk.) 8. Magyar Ökológus Kongresszus: elődások és poszterek összefoglalói. p. 172. (in Hungarian)

Ouanphanivanh N, Szigeti Z, Illyés Z (2010) Egyes hazai homok- és szénbányákban élő orchideák gombapartnereinek azonosítása molekuláris biológiai módszerekkel. Magyar Biológiai Társaság, Botanikai Szakosztály, 1440. szakülés, 2010. április 26., Budapest. Botanikai közlemények, 97 (1-2) p. 178. (in Hungarian)